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Signal Transduction, Chemotaxis, and Cell Aggregation in *Dictyostelium discoideum* Cells without Myosin Heavy Chain

DORIEN J. M. PETERS,* DAVID A. KNECHT,† WILLIAM F. LOOMIS,† ARTURO DE LOZANNE,‡
JAMES SPUDICH,‡ AND PETER J. M. VAN HAASSTERT*

*Cell Biology and Genetics Unit, Zoological Laboratory, University of Leiden, P.O. Box 9516, 2300 RA Leiden, The Netherlands; †Department of Biology, University of California San Diego, La Jolla, California 92093; and ‡Department of Cell Biology, Stanford University School of Medicine, Stanford, California 94305

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Dictyostelium discoideum cells have been generated that lack myosin heavy chain (MHC) due to antisense RNA inactivation of the endogenous mRNA or to insertional mutagenesis of the myosin gene. These cells retain chemotactic movement in gradients of the chemoattractant cAMP. Furthermore, cAMP does induce many biochemical and physiological responses in aggregative cells, including binding of cAMP to surface receptors, modification, and down-regulation of the receptor; activation of adenylate and guanylate cyclase, secretion of cAMP; and the association of actin to the Triton-insoluble cytoskeleton. Cells lacking MHC were found to have a requirement for bivalent cations in the medium for optimal chemotaxis and cell aggregation. © 1988 Academic Press, Inc.

INTRODUCTION

Dictyostelium discoideum cells have been transformed with a modified myosin heavy chain gene (*mhcA*) such that they lack the normal amount of myosin heavy chain (MHC). In one study a portion of the *mhcA* gene was fused in reverse orientation with a *D. discoideum* actin promoter. Less than 0.5% of the normal level of MHC accumulates at any time during development when the antisense *mhcA* RNA accumulates to greater than 10-fold the level of endogenous *mhcA* mRNA (Knecht and Loomis, 1987). In another study a vector containing a truncated *mhcA* inserted in the endogenous gene. The endogenous *mhcA* gene is disrupted, and cells synthesize only the truncated heavy meromyosin (HMM) but no MHC (De Lozanne and Spudich, 1987). Both cell lines that lack MHC have very similar phenotypes. Cells grow slowly when attached to a surface but do not grow in suspension. Cell division is impaired more strongly than nuclear division, and huge multinuclear cells arise frequently. Development is arrested at or shortly after the aggregation stage.

Cell aggregation in *D. discoideum* is mediated by chemotaxis to cAMP which is secreted by aggregation centers. Cyclic AMP binds to surface receptors; activation of these receptors induces the stimulation of many cellular processes, including the activation of adenylate and guanylate cyclase, the association of actin with the Triton-insoluble cytoskeleton, the uptake of Ca^{2+} and K^{+} ions, and the acidification of the medium (the physiology and biochemistry of cell aggregation has been reviewed by Devreotes, 1983; Gerisch, 1987; and Jans-

sens and Van Haastert, 1987). The analysis of mutants may allow one to assign the importance of these responses in chemotaxis. In this study the role of MHC in chemotaxis, cell aggregation, and transduction of cAMP signals was investigated.

Dictyostelium myosin is composed of two heavy chains of 240 kDa, and two each of two light chains of about 18 and 16 kDa (Clarke and Spudich, 1974). Myosin is a major protein of *D. discoideum* and is localized at the posterior of chemotactically stimulated cells (Yumara and Fukui, 1985). Stimulation of aggregation competent cells in suspension with the chemoattractant cAMP results in the rapid phosphorylation of the heavy chain and the 18-kDa light chain (Berlot *et al.*, 1985). Phosphorylation of the heavy chain inhibits thick filament assembly and actin-activated ATPase activity (Kuczmarski and Spudich, 1980). It is attractive to speculate that the changes in phosphorylation of myosin that occur by cAMP stimulation are involved in cell motility and chemotaxis. We report that *D. discoideum* transformants that lack MHC are chemotactically active and show essentially normal biochemical and physiological responses to cAMP.

MATERIALS AND METHODS

Strains and culture conditions. *mhcA* cells were generated by transformation of AX4 cells with plasmid pA6mhc6 which carries a fragment of the *mhcA* gene in the antisense orientation in vector pA6NPTII (Knecht and Loomis, 1987). HMM cells were generated by transformation of AX4 cells with plasmid pNEO-HMM140

which carries a fragment of the *mhcA* gene in vector pA6NPTII; this vector was inserted into the *mhcA* gene (De Lozanne and Spudich, 1987). Two control strains were used, AX4 and HL-300 which is a transformant of AX4 carrying the vector pA6NPTII, making it resistant to the antibiotic G418. Cells were grown on plastic support to confluency in HL5 medium containing 10 $\mu\text{g}/\text{ml}$ G418; AX4 cells were grown in the absence of G418.

Cells were collected by centrifugation at 100g for 3 min, washed twice in PB (10 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.5) or in MCPB (PB with 2 mM MgCl_2 and 0.2 mM CaCl_2), and starved in suspension for 5 hr at 22°C in PB or MCPB. Since cells of the four strains used are very different in size, all suspensions of cells were made by equal dilutions of the cell pellets, rather than using equal numbers of cells per milliliter. Protein contents in the suspensions were determined afterward and used to standardize the experimental data.

Cell aggregation and development. Cell aggregation and development was followed in small droplets (0.1 μl) containing about 300–500 cells which were deposited on hydrophobic agar (0.9% washed agar in PB or MCPB). Development was observed at 0.5- to 1-hr intervals.

Chemotaxis. Chemotaxis was measured with the small population assay (Konijn, 1970), with the modification that the hydrophobic agar was made in PB or MCPB.

Physiological and biochemical assays. After starvation for 5 hr in PB or MCPB, cells were washed and resuspended in PB. Binding of 5 nM [^3H]cAMP to cell surface receptors and down-regulation of the receptors by 10 μM cAMP were performed as described (Van Haastert, 1987). The covalent modification of the receptor by 1 μM cAMP results in a shift of its apparent molecular weight from 40 to 43 kDa on SDS-polyacrylamide gels (C. Klein *et al.*, 1985; P. Klein *et al.*, 1985). This modification was detected with anti-receptor antiserum on size-fractionated Western transfers as described by Klein *et al.* (1987) with some modifications for staining the receptor bands (Snaar-Jagalska *et al.*, 1988).

Cyclic GMP levels were measured after stimulation of cells with 0.1 μM cAMP with a sensitive radioimmunoassay (Van Haastert and Van der Heiden, 1983). Total and extracellular cAMP levels were determined with an isotope-dilution assay after stimulation of cells with 2 μM 2'-deoxy-cAMP and 5 mM dithiothreitol (Van Haastert, 1984).

The association of actin to the Triton-insoluble cytoskeleton was determined as described by McRobbie and Newell (1983), with the following modifications. Cells were lysed for 30 min at 0°C in 1% Triton X-100, 10 mM KCl, 10 mM imidazole, 10 mM EGTA, pH 7.0. Samples were centrifuged for 3 min at 10,000g, and the pellet was

washed twice with lysis buffer and finally resuspended in sample buffer (Laemmli, 1970). After electrophoresis of the cytoskeletons by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/Page), the gel was stained with 0.05% (w/v) Coomassie brilliant blue R-250 in 25% isopropanol and 10% acetic acid, and destained with 10% methanol in 10% acetic acid. Then the actin band was excised, and the stain was eluted in 3% SDS in 50% isopropanol by shaking at 37°C overnight (Ball, 1986). The extinction of the eluate was determined at 595 nm in a Beckman spectrophotometer.

The data shown are the means of two to four experiments; each experiment had at least one of the control strains (usually strain HL-300).

RESULTS

Aggregation of starved *D. discoideum* cells is mediated by chemotaxis to cAMP. When about 500 cells are placed in small droplets on hydrophobic agar, they remain in a small area (0.1 μm^2) from which they cannot escape due to the hydrophobicity of the agar surface. Thus, the cell density is sufficiently high to induce aggregation, but harmful waste products cannot accumulate to high concentrations; so delicate strains are in an optimal environment. Using this assay, we have observed that about 30% of *D. discoideum* mutants that do not aggregate on normal starvation plates can be induced to aggregate.

Cell Aggregation and Development

Control AX4 cells and control transformed cells, both containing normal amounts of MHC, aggregate on hydrophobic agar within about 13 hr (Table 1). The aggregation time and further development of these strains is not much dependent on the addition of bivalent cations to the agar. In contrast, the transformed *HMM* cells containing only heavy meromyosin and *mhcA* cells lacking MHC aggregate very poorly on hydrophobic agar in the absence of the bivalent cations Mg^{2+} and Ca^{2+} . Aggregation of these cell lines lacking MHC is improved considerably by including 0.2 mM CaCl_2 and 2 mM MgCl_2 in the agar. Cell aggregation is then only 1–2 hr later than in control cells and aggregation proceeds with streams and pulsatile movement of cells to the aggregation center with a periodicity of about 5 min. Although cell aggregation can be restored by bivalent cations, further development is still blocked beyond the formation of a tip on the aggregate.

Chemotaxis

Chemotaxis was quantified by placing small droplets containing different cAMP concentrations on the hy-

TABLE 1
CELL AGGREGATION AND DEVELOPMENT

	AX4	HL-300	mhcA	HMM
Content of MHC	+	+	<0.5%	Truncated
Development on PB	f	f	(a)	(a)
Development on MCPB	f	f	a	a
Time to aggregation on MCPB (hr)	13	13	14	15
Pulse interval on MCPB (min)	5.1	ND	5.3	4.9

Note. Small droplets (0.1 μ l) containing about 300–500 cells were deposited on hydrophobic agar (0.9% washed agar in PB or MCPB; PB stands for 10 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.5, and MCPB stands for PB with 2 mM MgCl_2 and 0.2 mM CaCl_2). Cells were incubated at 22°C and observed at 0.5- to 1-hr intervals. The time to aggregation is defined as the period after which 50% of the droplets had at least one aggregate. Time-lapse films were made when aggregation was in full progress, and the periodicity at which cells enter the aggregation center was determined. The symbols used have the following meanings: (a), some aggregation without streams; a, normal aggregation with streams, a small elevation resembling a tip was visible on the aggregate; f, complete fruiting body; ND, not determined.

drophobic agar close to the droplets with amoebae. The distribution of cells within the amoebal population was observed with about 5 min intervals. Transformants lacking MHC react poorly to the cAMP gradients in the absence of added bivalent cations (data not shown). In the presence of bivalent cations, however, the MHC-lacking cells have normal appearance and show essentially the same chemotactic response to cAMP as wild-type cells (Fig. 1). Control cells react chemotactically to cAMP, independent of the addition of bivalent cation to the agar (data not shown).

Development of Cell Surface cAMP Receptors

The role of bivalent cations on the acquisition of the aggregation competent stage was investigated. Cells were starved in phosphate buffer in the absence or presence of 2 mM MgCl_2 and 0.2 mM CaCl_2 , washed, and resuspended in phosphate buffer without bivalent cations, and the level of cAMP binding to surface receptors was determined. The transformant cells lacking MHC have essentially normal levels of cAMP receptor whether they are starved in the absence or presence of bivalent cations (Table 2). These results suggest that cells lacking MHC develop cell surface receptor independent of the addition of bivalent cations to the extracellular medium.

Alterations of the Surface cAMP Receptor

Treatment of *D. discoideum* cells with 10 μM cAMP for 15 min induces a loss of [^3H]cAMP-binding activity (Klein and Juliani, 1977; Van Haastert, 1987). This loss

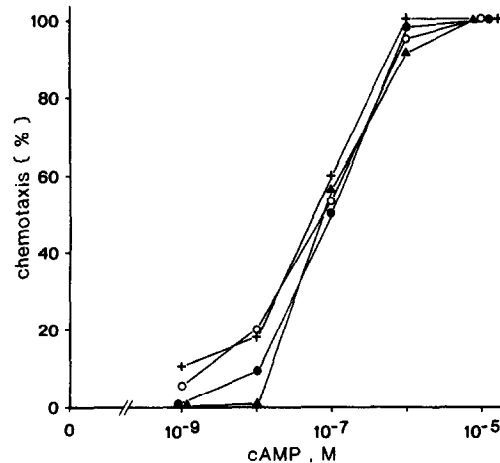


FIG. 1. Chemotaxis. The chemotactic activity was tested with the small population assay on hydrophobic agar containing MCPB (phosphate buffer with Mg^{2+} and Ca^{2+}). The chemotactic response of axenically grown cells is somewhat less than the response that we have observed repeatedly in wild-type NC4 cells. The difference appears to be due to the method of cell growth. Other data (not shown) of chemotaxis on agar without $\text{Mg}^{2+}/\text{Ca}^{2+}$ indicate very poor chemotaxis in mhcA and HMM cells and normal chemotaxis in AX-4 and HL-300 cells. (+) AX-4; (○) HL-300; (●) mhcA; (▲) HMM.

is essentially identical in control cells and in the cells that lack MHC (Table 2). This treatment of cells with cAMP also induces the covalent modification of the receptor as detected with the anti-receptor antiserum on Western transfers of size-separated proteins (Klein *et al.*, 1987). The covalent modification of the receptor was observed in all cell lines (Table 2).

Cyclic AMP and cGMP Response

All cell lines show a similar accumulation of cGMP levels after stimulation with 0.1 μM cAMP (Fig. 2A).

TABLE 2
cAMP RECEPTORS

	cAMP-binding (fmole/mg protein)			
	AX4	HL-300	mhcA	HMM
Starvation in MCPB	316	362	356	313
Starvation in PB	283	355	308	348
After down-regulation	33	26	47	46
Receptor modification	+	+	+	+

Note. Cells were starved in suspension in phosphate buffer in the absence (PB) or presence (MCPB) of 2 mM MgCl_2 and 0.2 mM CaCl_2 for 5 hr. All cells were washed in PB, and [^3H]cAMP binding was measured in PB. Down-regulation of cAMP receptors was induced in PB with 10 μM cAMP and 5 mM dithiothreitol for 15 min. [^3H]cAMP binding was measured in thoroughly washed cells. Receptor modification was induced in PB with 1 μM cAMP and 10 mM dithiothreitol for 5 min and was observed in all cell lines on Western transfers of size-separated proteins with the anti-receptor antiserum (Klein *et al.*, 1987).

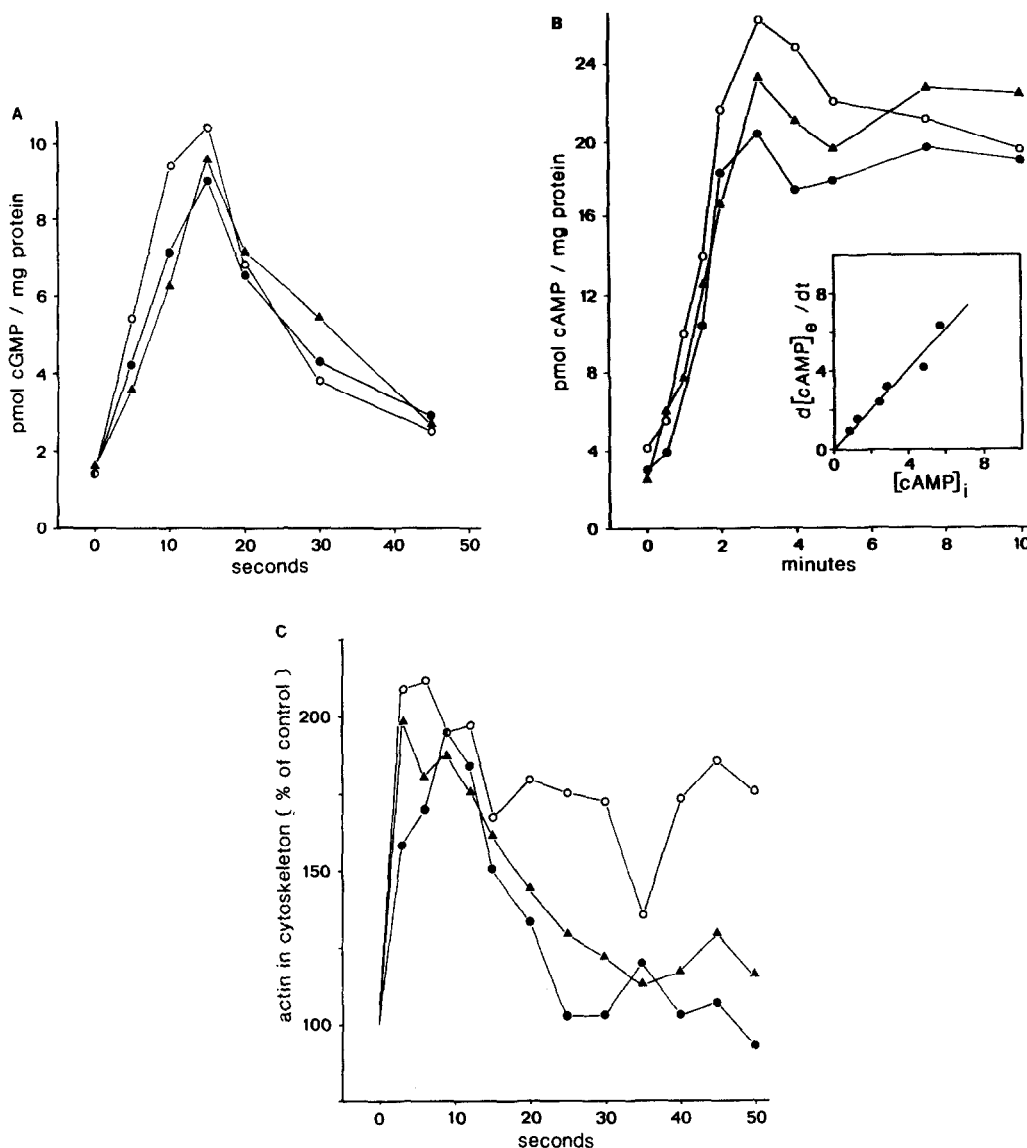


FIG. 2. cAMP-induced responses. Cells were starved in suspension in PB for 5 hr, washed, and resuspended in this buffer at a density of $2-6 \times 10^7$ cells/ml. (A) cGMP accumulation after stimulation with $0.1 \mu M$ cAMP. (B) Total cAMP accumulation after stimulation with $2 \mu M$ 2'-deoxy-cAMP and $5 mM$ dithiothreitol. Inset, the total and extracellular cAMP concentration was determined at various time moments after stimulation of mhcA cells. The intracellular cAMP concentration ($[cAMP]_i$ in pmole/mg protein) and the secretion rate ($d[cAMP]_e/dt$ in pmole/mg protein/min) were calculated. The slope of this curve yields the coupling constant of cAMP secretion. (C) The triton insoluble cytoskeletons were isolated after stimulation with $0.1 \mu M$ cAMP, proteins were separated by SDS-polyacrylamide gels, and the content of actin was determined. (○) HL-300; (●) mhcA; (▲) HMM.

The accumulation of total cAMP levels is also very similar after stimulation of the four cell lines with the receptor agonist 2'-deoxy-cAMP in the presence of the phosphodiesterase inhibitor dithiothreitol (Fig. 2B). In wild-type cells, the intracellularly produced cAMP is secreted; the secretion rate is proportional to the intracellular cAMP concentration with a coupling constant of about 1 min^{-1} (Dinauer *et al.*, 1980, Van Haastert, 1984). Total and extracellular cAMP levels were measured after stimulation of mhcA cells with 2'-deoxy-cAMP, and the intracellular cAMP levels were calcu-

lated, as well as the secretion rate. The secretion rate as a function of the intracellular cAMP concentration is linear with a slope of 1 min^{-1} (inset, Fig. 2B), indicating that the mechanism by which *D. discoideum* cells secrete cAMP is also not dependent on the presence of MHC.

Association of Actin to the Triton-Insoluble Cytoskeleton

Cyclic AMP induces the association of actin to the Triton-insoluble cytoskeleton, probably by polymeriza-

tion of actin filaments and extension of actin filaments at the barbed ends (McRobbie and Newell, 1985). This association is rapid after addition of cAMP and is generally followed by a second and third maximum. Triton-insoluble cytoskeletons were collected after stimulation with cAMP of control cells and transformants lacking MHC. Proteins were separated by SDS-polyacrylamide gel electrophoresis and the actin content was quantified (Fig. 2C). In control cells cAMP induces a twofold accumulation of actin in the cytoskeleton with a maximum at about 5 sec; the second and third maxima at 20 and 45 sec were not always clearly recognizable in our experiments (Fig. 2C). The transformed cells that lack MHC clearly show the rapid association of actin with the Triton-insoluble cytoskeleton (Fig. 2C).

These results indicate that transmembrane signal transduction is relatively normal in *D. discoideum* transformants that lack MHC and that signal transduction does not depend on elevated levels of bivalent cations.

DISCUSSION

D. discoideum cells that have less than 0.5% of the normal amount of MHC or that have only HMM grow slowly and form huge multinuclear cells (Knecht and Loomis, 1987; De Lozanne and Spudich, 1987). These cells aggregate only slightly later than control cells when placed on hydrophobic agar with the bivalent cations Mg^{2+} and Ca^{2+} in the medium. Increased levels of either Mg^{2+} or Ca^{2+} are beneficial to cell aggregation in MHC-lacking cells but are not essential for cell aggregation of control cells.

The requirement of chemotaxis and cell aggregation for bivalent cations is not fully understood. MHC-lacking cells develop normal levels of cell surface cAMP receptors, independent of the presence of bivalent cations during starvation. Furthermore, the intracellular accumulation of cGMP and the association of actin to the Triton-insoluble cytoskeleton are also not strongly dependent on higher levels of bivalent cations. These responses have been correlated with chemotaxis in *D. discoideum* (Van Haastert *et al.*, 1982; McRobbie and Newell, 1983). It is unlikely that MHC-lacking cells require higher levels of bivalent cations to compensate for reduced uptake of these ions, since the actin response, which is dependent on the liberation of intracellular Ca^{2+} ions, is relatively normal in MHC-lacking cells irrespective of the addition of bivalent cations. It is possible that bivalent cations stabilize membrane structures; this stabilization is essential for optimal cell locomotion of MHC-lacking cells on a solid support, but not for cAMP-induced responses in suspension.

In a small population assay we have seen that these cells have a relatively normal chemotactic behavior. In

this assay the movement of a population of *Dictyostelium discoideum* cells to the source of chemoattractant is detected. When individual cells are followed, however, MHC-lacking cells are observed to have a reduced capacity to fine tune locomotion in cAMP-gradients, such that chemotaxis is not optimal (Wessels *et al.*, 1988). A large portion of *mhcA* and HMM cells are multinucleated and up to 10-fold larger than control cells. Time-lapse cinematography reveals that these large cells extend many pseudopods at the same time. Translocation of large cells is less effective than translocation of small cells, presumably because the pseudopods are extended in all directions. When cells are placed in a gradient of cAMP, however, the huge cells make only one or two pseudopods and these pseudopods point in the direction of the cAMP gradient. Polarized cells are thus formed with a length up to 50 μm . These observations imply that the organization of cellular motility and the integration of chemotactic signals have not yet reached their limits in the largest cells. It also suggests that the number of nuclei is not decisive for the efficiency of chemotactic movement. It is intriguing how these giant cells are able to coordinate pseudopod formation during chemotactic movement.

Although the frequency of pseudopod formation is relatively normal, the initial area, rate of expansion, and final area of pseudopods are roughly half that of normal cells, suggesting that the MHC is involved in the fine regulation of pseudopod formation (Wessels *et al.*, 1988). Yumura and Fukui (1986) proposed that the native force for cell mobility is generated by contraction of myosin in the posterior cortex, followed by an extension of the hyaline cap with formation of a tight actin meshwork in the anterior part of the cell. It is possible that cells lacking MHC are still able to make pseudopods by actin polymerization but they have lost the contraction of myosin in the posterior cortex. This might be the cause of a decreased orientation and extension of pseudopods and orientation of the cell.

It was unexpected to find that MHC has such a limited function during cell locomotion (Wessels *et al.*, 1988), signal transduction (this report), and the activation of cell-type-specific genes (Knecht and Loomis, 1988). Mutants that lack MHC do not necessarily show that MHC is not functional during these processes in control cells. It is possible that the function of MHC is taken over by other proteins in MHC-lacking cells. The selection of secondary mutations that compensate for the lack of MHC could resolve this point. Nevertheless, it seems clear that the alterations that are induced by chemotactic signals in the localization (Yumura and Fukui, 1985) and phosphorylation (Berlot *et al.*, 1985) of the MHC molecule are not required for chemotaxis in *D. discoideum*.

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